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IMMUNOSUPPRESSION FOLLOWING EXPOSURE TO EXOGENOUS ESTROGENS

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INTRODUCTION

Physiological estrogens including estradiol and estrone can modulate immune responses. This is evidenced by the fact that female mice possess superior humoral mediated immunity (HMI) and inferior cell mediated immunity (CMI) compared to male mice and these effects can be negated by castration (Ahlquist, 1976). Other studies demonstrating that exogenously administered estrogens and androgens exert negative regulatory influences on CMI and HMI, respectively, support these findings (Fujii et al., 1975; Thompson et al., 1967). These observations may be of particular importance to environmental toxicology since many xenobiotics, natural products and potential environmental pollutants possess estrogenic activity and demonstrate specific binding to estrogen receptors (Katzenellenbogen et al., 1980). While most of these chemicals have relatively weak estrogenic activity compared to estradiol, there is evidence that either chronic exposure or acute exposure to weak estrogens which are not readily excreted (e.g. kepone) may lead to abnormal reproductive development and potential neoplasia (Eroschenko and Palmiter, 1980). Other compounds with weak estrogenic activity include polychlorinated biphenyls, o,p'-DDT, and methoxychlor. Some natural products that demonstrate binding affinity for the estrogen receptor are the flavones genistin, mirestrol, and the fluorescent coumestrol. The mycotoxin zearalanone (P-1502) and its more potent metabolite zearalanol (P-1496) produced by the fungus Fusarium also show remarkable binding affinity for the receptor although fairly poor uterotrophic activity (Katzenellenbogen et al., 1978).

With this in mind we began examining the immunotoxicity of a variety of nonsteroidal and steroidal estrogenic compounds using a

comprehensive testing panel developed at the NIEHS (Dean et al., 1982). Initially, diethylstilbestrol (DES), a nonsteroidal synthetic estrogen with potent estrogenic activity was examined. This compound has been employed as a therapeutic agent in humans as well as a growth promotant in livestock (McMartin, 1978). There is mounting evidence, however, that DES is potentially carcinogenic in humans and laboratory animals and has been associated with endometrial cancer, breast cancer, and vaginal adenocarcinoma (McLachlan, 1980). In mice, DES exposure suppresses specific immunity following either prenatal (Luster et al., 1979)., postnatal (Kalland, 1980a), or adult (Sljivic and Warr, 1973; Luster et al., 1980) exposure. In addition, both estradiol (Seaman et al., 1979a) and DES (Kalland, 1980b) inhibited natural killer cell activity, induced bone marrow myelotoxicity (Boorman et al., 1980), and activated macrophage functions (Boorman et al., 1980). In adult female mice exposed to DES, the effects on specific immune functions are predominantly on CMI rather than HMI (Luster et al., 1980), although this may not be the case following perinatal exposure (Kalland, 1980a). The effects of DES on host susceptibility to infectious agents and syngeneic tumor cell challenge are consistent with these immunological findings since host resistance assays which are dependent upon CMI and macrophage function for primary defense are markedly altered, while those assays dependent upon HMI are not. Thus, mice exposed to DES developed increased numbers of tumors following challenge with transplantable syngeneic tumor cells, increased mortality following challenge with Listeria monocytogenes, and decreased expulsion of adult worms from the gut following infection with Trichinella spiralis (Dean et al., 1980). In contrast to the above assays which are dependent upon CMI and macrophage functions for normal defense mechanisms, parameters which are dependent upon HMI are unaltered in DES exposed mice including susceptibility to infection with Plasmodium (P yoellii 17X) (Hayes, unpublished data) and encephalomyocarditis (EMC) virus (Munson, unpublished observations).

EXPERIMENTAL STUDIES

In more recent studies we have examined the effects of a variety of steroidal and non-steroidal compounds that demonstrate varying degrees of hormonal activity. Zearalanol is an estrogenic metabolite of the mycotoxin, zearalanone, which is produced from a variety of Fusarium species and has been proposed for use as a growth stimulant in certain species of livestock. The metabolite possesses approximately 17% the binding affinity of 17β-estradiol but less than 1% of its uterotrophic activity (Katzenellenbogen et al., 1978). As seen in Figure 1, it contains a phenolic ring structure in the A-ring providing the A-ring region structural similarities to DES and estradiol but not D-ring region. The phenolic ring structure is believed responsible for receptor binding affinity and specificity of estrogenic compounds.

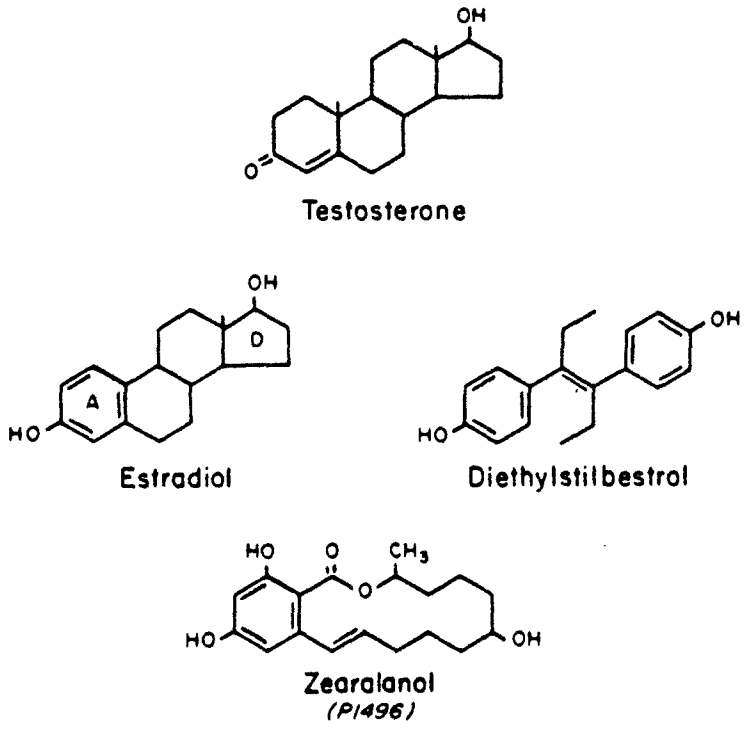

Figure 1. Structural formulae of testosterone, DES, zearalanol and estradiol.

While zearalanol is weakly estrogenic in the uterus, it readily reacts with liver estrogen receptors (Powell-Jones et al., 1981). Testosterone, in contrast, contains D-ring region, but not A-ring region structural similarities to estradiol and DES and appears to be selective for B-lymphocyte functions rather than T-lymphocyte functions (Fujii et al., 1975). It was hoped that by examining structure-activity relationships of these compounds, with respect to the immune system, some insight into the relationship between estrogen-induced immune suppression and hormonal activity would be gained.

For purposes of this presentation only representative parameters of immune function will be presented. Chemically-induced macrophage activation was examined using the growth inhibition assay which determines the capacity of macrophage to inhibit the in vitro growth of leukemia target cells (Dean et al., 1978). In this

instance we examined the ability of plastic adherent peritoneal cells to inhibit the growth of MBL-2 leukemia target cells (20:1 macrophage to target cell ratio). The percent inhibition of target cell proliferation by cells derived from control or chemically exposed mice is represented by ^{3}H-TdR incorporation into target cells and referred to as percent cytostasis. This is calculated from a formula:

$$\% \text{ Cytostasis} = \left(1 - \frac{\text{CPM MBL-2 + MO from treated mice}}{\text{CPM MBL-2 + MO from control mice}}\right) \times 100$$

Lymphocyte functions were determined by quantitating blastogenic responses of spleen cells following activation with T- and B-cell mitogens as well as allogeneic leukocytes. These assays are considered in vitro correlates of immune functions and were described in another chapter (see Chapter by Archer) and in greater detail elsewhere (Luster et al., 1982).

Bone marrow functions were evaluated by quantitating both cellularity and hematopoietic stem cell proliferation as performed in our laboratory (Boorman et al., 1980). Stem cell proliferation was determined by enumerating colony growth following injection of marrow cells into irradiated recipients using the colony forming unit-spleen (CFU-S) assay originally described by Till and McCulloch (1961).

RESULTS AND DISCUSSION

As shown in Table 1, exposure to either DES or estradiol caused severe thymic atrophy while compounds with less estrogenic activity (e.g. zearalanol) had only slight effects. Histologically, the thymus was characterized by severe depletion of cortical lymphocytes in DES or estradiol treated mice. Interestingly, this atrophy is histologically reversible since a normal appearing thymus was evident within 2 to 3 weeks following cessation of exposure to DES (Boorman et al., unpublished data). Cell mediated immunity as represented in Table 1 by lymphoproliferation to the polyclonal T-cell mitogen PHA and to allogeneic leukocytes in the MLC response was suppressed in mice exposed to DES and 17β-estradiol. Estrogenic compound caused a slight increase in LPS-induced B-cell activation, although diminished responses were evident in testosterone treated mice. In this respect, earlier studies indicated that testosterone as well as other androgens primarily affects the B-lymphocyte population as evidenced by depressed antibody plaque forming cell responses (Fujii et al., 1975). The suppression of plaque forming cell responses that we and others have reported following estradiol or DES exposure (Luster et al., 1980; Sljivic and Warr, 1973) represents a delayed onset of peak antibody responses probably induced by increased sequestering of antigen by activated macrophages (Sljivic and Warr, 1973; Bick et al., 1982).

TABLE 1. THYMIC ATROPHY AND PROLIFERATIVE RESPONSES OF SPLENIC LYMPHOCYTES IN HORMONE TREATED MICE

TREATMENT[a]	THYMIC ATROPHY (% CHANGE)	^{3}H-TdR INCORP. (nCPM x 10^{-3})[b] (% CHANGE)		
		PHA	LPS	MLC
CORN OIL	-	34 ± 3	22 ± 3	38 ± 5
DES	79↓**	20 ± 1** (41↓)	25 ± 3 (14↑)	13 ± 3** (66↓)
17β-ESTRADIOL	55↓**	23 ± 3** (32↓)	30 ± 4** (34↑)	20 ± 2** (47↓)
ZEARALANOL	25↓*	33 ± 2 (3↓)	24 ± 2 (9↑)	34 ± 6 (11↓)
TESTOSTERONE	14↓	34 ± 3 (0)	16 ± 4* (27↓)	34 ± 3
PROGESTERONE	4↓	28 ± 2 (18↓)	21 ± 1 (5↓)	ND

[a] Adult female mice were administered 2.8 μmoles of hormone subcutaneously in corn oil over a 5 day period. Each value represents mean ± SEM of 7 mice/group.

[b] Mean ± SEM of 7 mice/group. Data are expressed as net CPM = counts per minute (CPM) in stimulated cultures - CPM in cultures without stimulator.

* $P<0.05$ vs controls.

** $P<0.01$ vs controls.

Table 2 presents bone marrow and macrophage response data in mice exposed to equimolar concentrations of various steroidal and nonsteroidal hormones. As with lymphoproliferative responses, the most pronounced effect on bone marrow and macrophage functions occurs following exposure to the most estrogenic compounds, mainly DES and estradiol. However, zearalanol which as mentioned earlier is considerably less estrogenic than DES or estradiol was equally effective in activating macrophages and suppressing splenic colony forming units (CFU-S) numbers, but had no effect on bone marrow cellularity. Neither progesterone nor testosterone had any

demonstrable effect on bone marrow or macrophage functions. The data in Tables 1 and 2 provide evidence of a chemical disassociation indicating that regulation of estrogen-induced immunotoxicity is a complex event.

TABLE 2. BONE MARROW FUNCTIONS AND MACROPHAGE ACTIVITY IN HORMONE TREATED FEMALE MICE

	BONE MARROW		MACROPHAGE CYTOSTASIS	
TREATMENT[a]	CELLULARITY/ FEMUR x 10^6	CFU-S/ 5 x 10^4 CELLS	^{3}H-TdR INCORP. IN MBL-2 TARGET CELLS (CPM ± SEM x 10^{-3})	PERCENT CYTOSTASIS
CORN OIL	20.5 ± 0.9	16.7 ± 0.3	75 ± 4	-
DES	14.3 ± 1.1* (30↓)	10.2 ± 0.4* (39↓)	29 ± 7*	62%
17β-ESTRADIOL	14.6 ± 0.9* (29↓)	13.5 ± 0.3* (19↓)	30 ± 3*	60%
ZEARALANOL	17.8 ± 1.3 (13↓)	11.6 ± 0.3* (31↓)	34 ± 6*	54%
TESTOSTERONE	17.2 ± 1.3 (16↓)	15.9 ± 0.2 (5↓)	87 ± 7	+18%
PROGESTERONE	17.2 ± 1.4 (16↓)	16.1 ± 0.2 (4↓)	62 ± 9	17%

[a] Adult female mice were administered 2.8 μmoles of hormone subcutaneous in corn oil over a 5 day period. Each value represents the mean ± SEM of at least 6 mice/group.

* P<0.01 vs controls.

To determine whether the immunotoxicity was mediated through estrogen receptors, we examined the activity of several estrogen antagonists. Two of the most studied non-steroidal estrogen antagonists, when administered prior to estrogen exposure, are derivates of triphenylethylene and diphenyl (dihydro- or tetrahydro-) naphthalene, represented by Tamoxiphen and Nafoxidine, respectively (Sutherland and Murphy, 1982). These compounds, by themselves, are slightly agonistic but when administered along with estrogens demonstrate partial antagonism with respect to various estrogenic activities by neutralizing the estrogen receptor (Clark et al., 1980). As summarized in Table 3, administration of either Tamoxiphen or Nafoxidine had a significant effect on estrogen-induced thymic atrophy. However, almost complete inhibition of estrogen myelotoxicity and depressed lymphocyte blastogenesis occurred when mice were

pre-exposed to these antiestrogens. We have also recently demonstrated the presence of a specific estrogen-binding component present in relatively high concentrations in the bone marrow as well as confirmed earlier studies by Gillette and Gillette (1978) demonstrating the presence of specific estrogen receptors in thymus cytosol (data not shown). These studies suggest that the initial events associated with immunotoxicity are mediated through a specific estrogen receptor similar to that described in the uterus.

TABLE 3. THE EFFECT OF ANTIESTROGENS ON ESTROGEN-INDUCED IMMUNOTOXICITY[a]

ANTIESTROGEN TREATMENT	ESTROGEN EXPOSURE	PERCENT CHANGE FROM CONTROLS			
		THYMIC ATROPHY	BONE MARROW CELLULARITY	BONE MARROW CFU-S	LYMPHOCYTE PHA RESPONSE
VEHICLE	VEHICLE	-	-	-	-
TAMOXIPHEN	VEHICLE	40↓**	7↓	2↓	17↓
TAMOXIPHEN	ESTRADIOL	54↓**	5↓	1↓	9↓
NAFOXIDINE	VEHICLE	25↓*	1↓	2↓	13↓
NAFOXIDINE	ESTRADIOL	39↓**	8↓	2↓	6↓
VEHICLE	ESTRADIOL	64↓**	21↓**	18↓**	40↓**

[a] Mice were given 17 μmoles of antiestrogen over a 3-day period. 17β-estradiol (3.8 μmoles) was administered during the last two days of antiestrogen treatment. Mice were tested 4 days later. Each value represents mean ± SEM of 6 mice/group.

* $P<0.05$ vs controls (vehicle).

** $P<0.01$ vs controls (vehicle).

Studies by Stimson and Hunter (1980) suggested that selected immune effects induced by estrogens are mediated through the thymus following estrogen treatment. This was suggested by the fact that sera from estrogen treated rats, but not estrogen exposed thymectomized (Tx) rats, were capable of affecting several in vitro correlates of immune function. In experiments performed in our laboratory, adult female mice were surgically Tx, subsequently exposed to DES or 17β-estradiol and examined in the abbreviated testing panel (Table 4). Unlike non-Tx estrogen treated mice, neither macrophage activation nor suppression of CFU kinetics occurred in DES or estradiol exposed Tx mice. Tx, however, failed to

influence estrogen-induced bone marrow hypocellularity or suppression of lymphocyte function (MLC response). PHA responses of splenic lymphocytes in non-estrogen treated controls were significantly depressed as a result of Tx complicating interpretation of the PHA data. In recent studies, response in sham-Tx, estrogen exposed mice were found to be indistinguishable from estrogen exposed non-Tx mice (data not shown). These data demonstrate that selected immunomodulatory effects induced by estrogens can be relegated to the thymus. Although the manifestations of the estrogen/thymus interplay are obviously complex and probably not responsible for CMI suppression, it is interesting to note that Tx induced a similar bimodal disassociation of immune effects as observed in mice exposed to zearalanol. That is, zearalanol exposure altered macrophage activity and CFU kinetics without affecting lymphocyte blastogenesis or bone marrow cellularity. Tx inhibited the ability of estrogens to influence macrophage activity or CFU kinetics. The molecular events associated with these interactions are unknown but may be initiated by specific binding of the compound to cytosolic receptors present in various target cells (e.g. stem cell) or secondary target organ (i.e. thymus), thus representing a secondary hormonal effect. With respect to the latter, it is well recognized that the thymus is capable of regulating many immune functions including CFU kinetics through "thymic factors", although this regulation usually reflects positive influences (Goodman et al., 1978).

TABLE 4. EFFECT OF THYMECTOMY ON ESTROGEN-INDUCED IMMUNE ALTERATIONS[a]

PARAMETER	NONTHYMECTOMIZED			THYMECTOMIZED		
	CONTROL	DES	ESTRADIOL	CONTROL	DES	ESTRADIOL
MACROPHAGE						
Cytostasis (CPM x 10^3)	63	30*	36*	47	91*	101**
(% Cyto)		(52)	(43)	(75)	(+33)	(+60)
BONE MARROW						
Cellularity (x 10^5)	20 ± 2	12 ± 1**	14 ± 2**	19 ± 1	12 ± 1**	13 ± 1**
CFU-S/5 x 10^4 cells	17 ± 0.3	12 ± 0.2**	11 ± 0.2 **	18 ± 0.1	17 ± 0.3	17 ± 0.3
LP						
PHA (ncpm x 10^3)	25 ± 2	12 ± 2**	16 ± 1**	11 ± 1*	8 ± 1**	12 ± 3**
MLC (ncpm x 10^3)	19 ± 5	6 ± 1**	10 ± 1*	15 ± 1	5 ± 1**	7 ± 1**

[a] Mice were surgically Tx and treated with either DES (4.0 mg/kg) or estradiol (16 mg/kg) two days later for 5 consecutive days. Three days following the last treatment animals were tested.

* $P<0.05$ vs controls.

** $P<0.01$ vs controls.

Mice administered estrogenic compounds demonstrate a marked increase in susceptibility to infection with Listeria monocytogenes (Dean et al., 1980). The mechanisms responsible for the exquisite sensitivity of this system are unknown but appear primarily related to a combination of depressed cell mediated immunity and/or defective bactericidal activity in estrogen-induced inflammatory macrophages (Luster et al., unpublished data). The data from Table 5 suggest that surgical Tx prior to exposure to estradiol is capable of protecting mice from estrogen-induced increased susceptibility to infection. Since surgical Tx protects mice from estrogen-induced myelotoxicity and induction of activated macrophages, it would appear that the effects of estrogens on either or both of these parameters may be at least partially responsible for the decreased resistance to Listeria. Preliminary studies in our laboratory indicate that the mechanism responsible for the estrogen-induced alteration in susceptibility is primarily related to depressed cell mediated immunity and/or defective macrophage bactericidal activity rather than bone marrow myelotoxicity (Luster et al., unpublished data).

TABLE 5. LISTERIA INFECTION IN ADULT-THYMECTOMIZED ESTROGEN TREATED MICE

Treatment	Dead/Total	% Mortality
Sham	0/14	0
Corn Oil	2/13	15
Estradiol	8/13*	62*
Corn Oil ATX	1/13	8
Estradiol ATX	2/14	14

Sham or adult thymectomized (ATX) mice were administered corn oil or 0.1 mg/kg 17β-estradiol daily for 5 consecutive days. Four days following the final exposure all mice were intravenously injected with 5×10^4 viable Listeria monocytogenes.

*Significantly different from corn oil control ($P<0.05$) by Chi-square analysis.

SUMMARY

Exposure to pharmacological dosages of estrogens, including 17β-estradiol and DES, selectively affects immune responses. This immunologic profile is consistent with myelotoxicity, suppression of cell mediated immunity (CMI), and induction of inflammatory macrophages. Modulation of several of these functions is mediated through the thymus, since thymectomy abolishes estrogen-induced macrophage activation, inhibition of CFU-kinetics and increased susceptibility to Listeria infection, but does not inhibit depression of CMI or bone marrow cellularity. These effects can also be

disassociated chemically as zearalanol, an estrogenic mycotoxin, influences macrophage activity and CFU kinetics without affecting CMI. This may be due to structural differences since zearalanol is similar to DES and estradiol in the A-ring region but not D-ring region of the molecule. Underlying this explanation is the demonstration that many of these effects are apparently mediated through estrogen receptors, as indicated indirectly by inhibition with estrogen antagonist and the demonstration of estrogen receptors in thymus and bone marrow cell cytosol preparations. Thus, many of these selective effects may depend upon relative affinity to receptors as well as binding to the relevant target cell(s).

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